Amendments to the Drawings:

Please replace current Figures 3A-3C with the replacement Figures 3A-3C attached hereto as **Exhibit C.**

Sequence Listing:

Please insert into the application the Sequence Listing attached hereto as **Exhibit D.**

REMARKS

Claims 1-7, 37 and 57-61 are pending. Applicants have hereinabove amended claims 1, 7, 37 and 58 and have canceled claims 2-5 and 60-61 without disclaimer or prejudice to applicants' right to pursue the subject matter of these claims in the future. Support for the amendments to claims 1, 37 and 58 can be found in the specification as originally filed at, *inter alia*, page 11, line 31 to page 12, line 2, page 12, lines 25-32, and page 24, lines 24-25. Support for the amendment to claim 7 can be found in the specification as originally filed at, *inter alia*, page 12, lines 20-21. After entry of this Amendment claims 1, 6, 7, 37 and 57-59 as amended will be pending.

Objection to Specification

The July 22, 2009 Office Action objected to the disclosure of the specification because of the following informalities:
(1) The abbreviations PKR, Mx, ZAP and GAPDH should be spelled out in full for the first instance of use.
(2) There is an inconsistency between the identification numbers of the references cited in the text on page 28 and the numbers included in the Reference list on pages 30-31.

Applicants' Response

In response, Applicants herein provide replacement paragraphs to amend the specification.

Specifically, Applicants have amended the specification on page 1, line 23 to recite protein kinase R (PKR); on page 1, line 25 to recite myxovirus-resistance (Mx) proteins; on page 2, line 3 to recite Zinc-finger antiviral protein (ZAP) protein; and on page 8, line 9 to recite glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Additionally, some typographical errors were corrected in the replacement paragraphs.

Additionally, Applicants herein provide a replacement paragraph correcting the numbers referencing the cited publications listed on pages 30-31 of the subject specification.

Objection to Drawings

In the July 22, 2009 Office Action the Examiner objected to the drawing of Fig. 3B filed February 13, 2006 asserting that "CCCH Fingers" should be changed to "CCCH finger motifs" as set forth in the specification (p.7, lines 12 and 13).

Applicants' Response

In response, Applicants attach hereto as **Exhibit C** a replacement sheet for Figures 3A-3C, wherein the label of Fig. 3B has been revised.

Sequence Compliance

The July 22, 2009 Office Action contained a Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures (the "July 22, 2009 Notice") that indicates the application fails to comply with 37 C.F.R. §§1.821-1.825. Applicant attaches hereto a copy of the Notice as **Exhibit A.**

In response, applicant notes that the July 22, 2009 Notice set forth a time period to reply of one month from the issue date of the July 22, 2009 Notice. The July 22, 2009 Notice further indicated that extensions of time were available upon payment of a fee. In order to avoid paying an extension fee, applicant responded to the July 22, 2009 Notice within the required one-month period. The Examiner issued an October 22, 2009 Communication, a copy of which is attached hereto as **Exhibit B,** indicating that the reply was not fully responsive to the July 22, 2009 Office Action. However, applicant notes that the Amendment In Response To July 22, 2009 Notice To Comply, filed August 18, 2009, was filed in response to the Notice to Comply, as indicated in the Amendment, not the Office Action. Accordingly, applicant maintains that the Communication issued October 22, 2009 was not properly issued. If the Examiner's logic set forth in the July 22, 2009 Notice was followed, applicant would have been required to pay a two-month extension of time fee for the Sequence Listing Amendment if applicant had filed the response concurrently with a response to the Office Action at the three-month date, i.e. October 22, 2009. Applicant

further notes that this issue was pointed out to Examiner Liu in an October 15, 2009 telephone conference with Mr. Brian Amos of the undersigned's Office, which teleconference was initiated by Examiner Liu. Applicant therefore respectfully requests withdrawal of the October 22, 2009 Communication because of any adverse effect it would have on Patent Term Adjustment otherwise available to applicant.

Nevertheless, in order to advance prosecution, applicant is re-submitting as part of the current response amendments to conform the application with 37 C.F.R. §§1.821-1.825.

Applicant attaches hereto a paper copy Sequence Listing as **Exhibit D,** a Statement in accordance with 37 C.F.R. §1.821(f)and(g) as **Exhibit E**, and a computer readable format Sequence Listing as **Exhibit F**.

The computer readable format Sequence Listing and paper copy Sequence Listing contain no new matter as required by 37 C.F.R. §1.821 and 37 C.F.R. §1.825.

Applicant has amended the specification to include the appropriate references to the sequence identification numbers. Applicant has also added the appropriate sequence identification numbers to Figure 3B and a replacement sheet for Figures 3A-3C is attached hereto as **Exhibit C**.

Claims Rejected Under 35 U.S.C. §112, First Paragraph

In the July 22, 2009 Office Action the Examiner rejected claims 1-4, 6 and 7 as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner asserted that without setting forth a SEQ ID NO or indicating that the claimed protein is a full-length polypeptide from a certain species and/or reciting a biological function thereof, an "isolated ZAP protein" is drawn to genus of "ZAP proteins" including fragments or mutated variants of the full-length polypeptide, for which there is insufficient disclosure in the instant specification.

Applicants' Response

In response, applicants respectfully traverse the Examiner's rejection. However, without conceding the correctness of the Examiner's position, applicants have hereinabove amended claim 1 to recite a "mammalian Zinc-finger antiviral protein (ZAP) protein which (a) comprises four CCCH-type zinc finger motifs; and (b) when present in a mammalian cell infected with a retrovirus, binds to RNA of the retrovirus, so as to inhibit replication of the retrovirus in the cell" (emphasis added). In accordance with the Examiner's suggestion, applicants have limited the claims to the mammalian form of the ZAP protein that contains a structural motif of four CCCH-type zinc fingers and binds to RNA corresponding to a retrovirus to inhibit

replication of that retrovirus. Thus, the claims can no longer be construed to encompass ZAP proteins, including fragments or variants thereof, that are biologically inactive for the purpose of inhibiting viral replication, and Ramos et al. is no longer a relevant prior art reference.

Accordingly, applicants maintain the invention as now claimed meets the written description requirement and therefore respectfully request reconsideration and withdrawal of this ground of rejection.

Anticipation Rejection Under 35 U.S.C. §102(b)

The July 22, 2009 Office Action rejected claims 1 and 2 under 35 U.S.C. §102(b) as allegedly anticipated by Hatada et al. (US Patent No. 6251620 B1) because they teach a human "ZAP" protein called "ZAP NC" (col.2, line 30).

Claims 1 and 2 were also rejected under 35 U.S.C. §102(b) as allegedly anticipated by Babiyuchuk et al. (US 2001/0011381 A1) because they teach a purified human PARP which is a Zn-finger containing protein of ZAP family (class).

Applicants' Response

In response, applicants respectfully traverse the Examiner's rejection. However, without conceding the correctness of the Examiner's position, applicants have hereinabove amended claim 1 to recite a "mammalian Zinc-finger antiviral protein (ZAP) protein which (a) comprises four CCCH-type zinc finger motifs;

and (b) when present in a mammalian cell infected with a retrovirus, binds to RNA of the retrovirus, so as to inhibit replication of the retrovirus in the cell" (emphasis added). The limitation to a mammalian protein in conjunction with the recitation of structural features ("four CCCH-type zinc finger motifs") and functional features ("when present in a mammalian cell infected with a retrovirus, binds to RNA of the retrovirus, so as to inhibit replication of the retrovirus in the cell") unambiguously differentiates the claimed protein from the prior art.

In the July 22, 2009 Office Action, the Examiner asserted that Hatada et al. teach a "ZAP" protein (ZAP NC); however, in their disclosure, "ZAP" stands for "zeta-associated protein," which is a protein tyrosine kinase. *See,* Hatada et al., *Nature,* 1995 Sep 7; 377(6544):32-8, page 32, column 1, attached hereto as **Exhibit G**. The zeta-associated protein (ZAP) of Hatada et al. is not a mammalian Zinc-finger antiviral protein comprising four CCCH-type zinc finger motifs that binds to RNA corresponding to a retrovirus, so as to inhibit replication of the retrovirus in the cell. Thus, applicants' "Zinc-finger antiviral protein" is not anticipated by Hatada et al.'s "zeta-associated protein" and Hatada et al. should be removed as a prior art reference.

The Examiner also asserted that Babiychuk et al. teach a purified human PARP which is a Zn-finger containing protein of "ZAP family (class)." In their disclosure, "ZAP" stands for "Zn-finger poly(ADP-ribose) polymerase," which catalyzes the transfer of an ADP-ribose moiety derived from NAD+ (nicotinamide adenine dinucleotide), mainly to the carboxyl group of a glutamic

acid residue in the target protein, and subsequent ADP-ribose polymerization. *See,* Babiychuk et al. [0007] and [0054]. The Zn-finger poly(ADP-ribose) polymerase (ZAP) of Babiychuk et al. is not a mammalian Zinc-finger antiviral protein comprising four CCCH-type zinc finger motifs that binds to RNA corresponding to a retrovirus, so as to inhibit replication of the retrovirus in the cell. Thus, applicants' "Zinc-finger antiviral protein" is not anticipated by Babiychuk et al.'s "Zn-finger poly(ADP-ribose) polymerase" and Babiychuk et al. should also be removed as a prior art reference.

Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Obviousness Rejection Under 35 U.S.C. §103(a)

The July 22, 2009 Office Action rejected claims 1 and 3-5 under 35 U.S.C. §103(a) as allegedly unpatentable over Gao et al. (*Science* (2002, Sept. 6) 297, 1703-1706).

Applicants' Response

Initially, applicants note that the subject application claims the benefit of the August 13, 2003 filing date of U.S. Provisional Application No. 60/494,764 (hereinafter "the Provisional Application"). Applicants further note that claims 1, 6, 7, 37 and 57-61 are fully supported in the disclosure of the Provisional Application. Specifically, support for claim 1 can be found in the Provisional Application as originally filed at, *inter alia*, page 11, line 31 to page 12, line 2, page 12, lines 25-32, and page 24, lines 24-25. Support for claims 6 and 7 can be found in the Provisional Application as originally filed at, *inter alia*, page 13, lines 14-16. Support for claim 37 can be found in the Provisional Application as originally filed at, *inter alia*, page 15, lines 25-29. Support for claim 57 can be found in the Provisional Application as originally filed at, *inter alia*, page 15, line 30. Support for claim 58 can be found in the Provisional Application as originally filed at, *inter alia*, page 11, line 31 to page 12, line 2, page 15, line 32 to page 16, line 3. Support for claim 59 can be found in the Provisional Application as originally filed at, *inter alia*, page 16, lines 5-6. Support for claim 60 can be found in the Provisional Application as originally filed at, *inter alia*, page

16, lines 6-7. Support for claim 61 can be found in the Provisional Application as originally filed at, *inter alia*, page 16, lines 7-8.

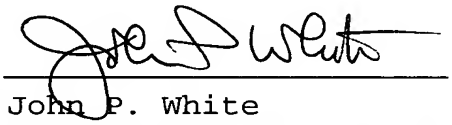
Applicant notes that Gao et al. is applicants' own publication and attaches hereto as **Exhibit H** a Declaration Under 37 C.F.R. §1.132 by Dr. Stephen P. Goff declaring that he and Dr. Guangxia Gao are the inventors of the invention in claims 1, 6-7, 37 and 57-61, and that the other author listed on Gao et al. (*Science* (2002, Sept. 6) 297, 1703-1706), i.e. Xuemin Guo, did not contribute to the conception of the invention in claims 1, 6-7, 37 and 57-61. Gao et al. is applicants' own work. Thus, Gao et al. is not prior art and cannot be used to support an obviousness rejection under 35 U.S.C. §103.

Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

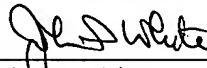
No fee, other than the $245.00 fee for a two-month extension of time, is deemed necessary in connection with the filing of this Amendment. However, if any other fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham LLP
30 Rockefeller Plaza
New York, New York 10112
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450



12/22/09

John P. White Date
Registration No. 28,678

EXHIBIT A

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, DC 20231
www.uspto.gov

APPLICATION NO. /CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
10568396	8/31/2006	GOFF ET AL.	67489-PCT-US/JPW/JW

EXAMINER
SAMUEL W. LIU

ART UNIT	PAPER
1656	20090709

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents

This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 C.F.R. § 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 C.F.R. §§ 1.821-1.825 for the reason(s) set forth on the attached Notice To Comply With Requirements For Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures. Applicant must comply with the requirements of the sequence rules (37 CFR 1.821 - 1.825) before the application can be examined under 35 U.S.C. §§ 131 and 132.

APPLICANT IS GIVEN ONE MONTH FROM THE DATE OF THIS LETTER WITHIN WHICH TO COMPLY WITH THE SEQUENCE RULES, 37 C.F.R.. §§ 1.821-1.825. Failure to comply with these requirements will result in ABANDONMENT of the application under 37 C.F.R. § 1.821(g). Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 C.F.R. § 1.136. In no case may an applicant extend the period for response beyond the six month statutory period. Direct the response to the undersigned. Applicant is requested to return a copy of the attached Notice to Comply with the response.

The addresses below are effective 5 June 2004. Please direct all replies to the United States Patent and Trademark Office via one (1) of the following:

1. Electronically submitted through EFS-Web (<http://www.uspto.gov/ebc/efs/downloads/documents.htm>, EFS Submission User Manual - ePAVE)
2. Mailed to:
   Mail Stop Sequence
   Commissioner for Patents
   P.O. Box 22313 1450
   Alexandria, VA 22313 1450
3. Hand Carry, Federal Express, United Parcel Service or other delivery service to:
   U.S. Patent and Trademark Office
   Mail Stop Sequence
   Customer Window
   Randolph Building
   401 Dulaney Street
   Alexandria, VA 22314

Any inquiry concerning this communication should be directed to Samuel Liu at telephone number (571)272-0949. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Andrew M. Wang, can be reached on 571-272-0811.

EXHIBIT B

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/568,396	08/31/2006	Stephen P. Goff	67489-PCT-US/JPW/JW	6298

23432 7590 10/22/2009

COOPER & DUNHAM, LLP
30 Rockefeller Plaza
20th Floor
NEW YORK, NY 10112

EXAMINER	
LIU, SAMUEL W	

ART UNIT	PAPER NUMBER
1656	

MAIL DATE	DELIVERY MODE
10/22/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
10568396	8/31/2006	GOFF ET AL.	67489-PCT-US/JPW/JW

COOPER & DUNHAM, LLP
30 Rockefeller Plaza
20th Floor
NEW YORK, NY 10112

EXAMINER	
SAMUEL LIU	
ART UNIT	**PAPER**
1656	20091015

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The reply filed on 8/21/09 is not fully responsive to the prior Office Action because of the following omission(s) or matter(s): Applicant has not adequately replied to the 112/1st rejection (written description), the 102 rejections and 103 rejections set forth in the Office action mailed 7/22/09. A request to hold a rejection in abeyance is not a proper response to a rejection. Rather, a request to hold a matter in abeyance may only be made in response to an OBJECTION or REQUIREMENTS AS TO FORM (see MPEP 37 CFR 1.111(b) and 714.02). Upon communication with Brian Ames on behalf of John White on 10/15/09, it was confirmed that applicants have not filed the response to the Office action with regard to the claims rejections as mentioned above; Applicants will file this response. It was also confirmed that applicants have only responded to the sequence compliance set forth in the office action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Samuel Liu whose telephone number is (571)272-0949. The examiner can normally be reached on Monday-Friday, 9 am to 5:30 pro. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Andrew Wang, can be reached on 571-272-0811. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

/SAMUEL LIU/
Examiner, Art Unit 1656

/ANAND U DESAI/
Primary Examiner, Art Unit 1656

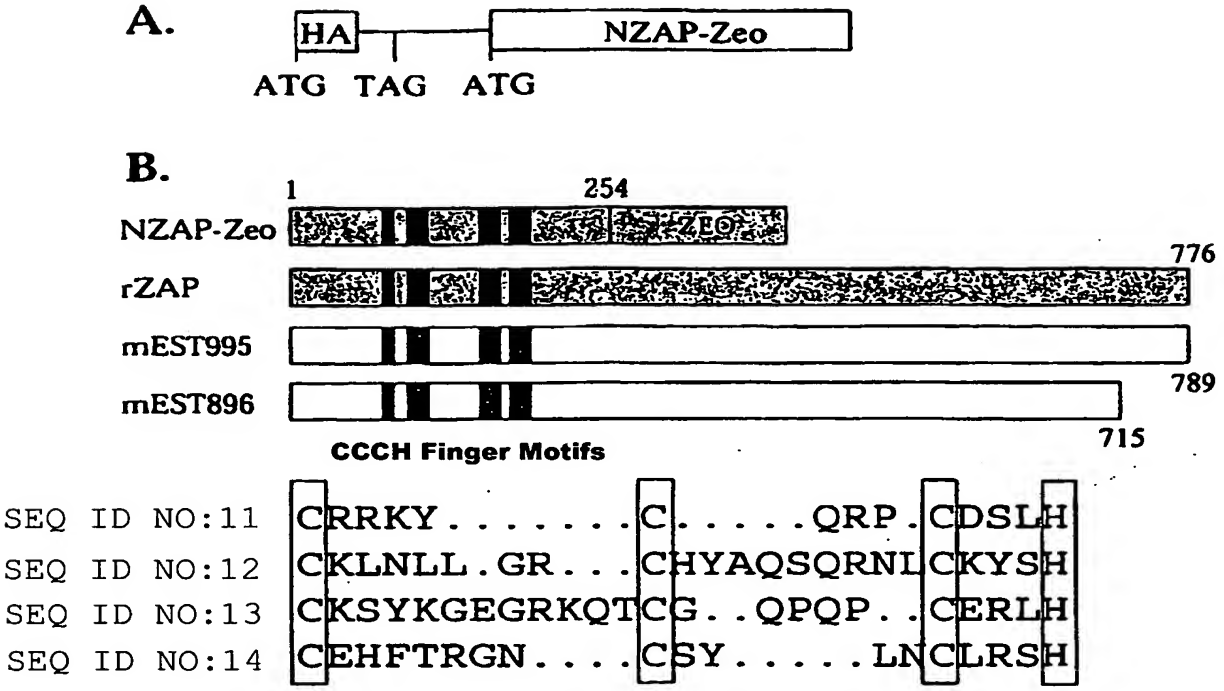
EXHIBIT C

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao



FIGS. 3A - C

EXHIBIT D

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

SEQUENCE LISTING

<110> THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK

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EXHIBIT E

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

Docket No. 0575/67489-PCT-US/JPW/BJA/MJP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Stephen P. Goff and Guangxia Gao	
International Application No.	:	PCT/US2004/026162	
Serial No.	:	10/568,396	Examiner : Liu, S.
Filed	:	August 31, 2006	Art Unit : 1656
For	:	ZAP PROTEIN AND RELATED COMPOSITIONS AND METHODS	

30 Rockefeller Plaza
New York, New York 10112
December 22, 2009

Mail Stop Sequence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

STATEMENT IN ACCORDANCE WITH 37 C.F.R. §§ 1.821(f) AND (g)

Pursuant to 37 C.F.R. §1.821(f), I hereby certify that the content of the Sequence Listing enclosed herewith as Exhibit D and the content of the computer readable form of the sequence listing enclosed herewith as Exhibit F are identical. Pursuant to 37 C.F.R. §1.825(a), I hereby certify that the Sequence Listing enclosed herewith as Exhibit D contains no new matter.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Michael Pearce
Cooper & Dunham LLP
30 Rockefeller Plaza
New York, New York 10112
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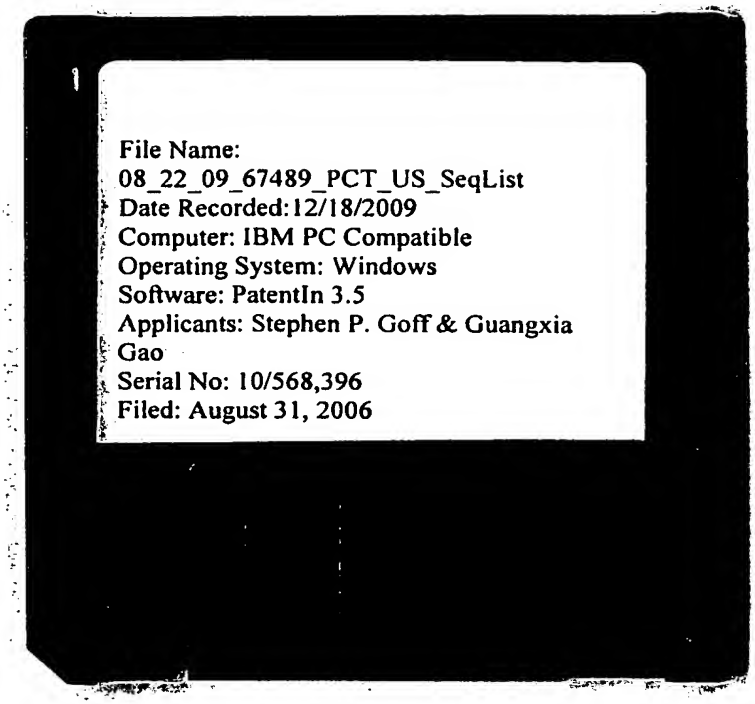
EXHIBIT F

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao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Applicants: Stephen P. Goff & Guangxia Gao
Serial No: 10/568,396
Filed: August 31, 2006

EXHIBIT G

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor

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The crystal structure of the tandem SH2 domains of human ZAP-70 in complex with a peptide derived from the ζ-subunit of the T-cell receptor reveals an unanticipated interaction between the two domains. A coiled coil of α-helices connects the two SH2 domains, producing an interface that constitutes one of the two critical phosphotyrosine binding sites. These and other unique features provide the molecular basis for highly selective association of ZAP-70 with the T-cell receptor.

T-CELL recognition of antigen-presenting cells initiates a cascade of intracellular processes that ultimately result in changes in gene expression, the production of secreted mediators, and cellular proliferation[1,2]. This recognition is mediated by the T-cell antigen receptor (TCR), which consists of the antigen-binding α- and β-subunits, the CD3 complex of γ-, δ- and ε- chains, and the ζ-homodimer. With the exception of α and β, the intracellular portion of each subunit includes one to three amino-acid sequences that contain the motif $YXX(L/I)X_{(7-8)}YXX(L/I)$, where X is variable[3]. After receptor stimulation, these immunoreceptor tyrosine activation motifs (ITAMs) become phosphorylated on tyrosine residues and, in this modified form, provide binding sites for downstream signalling proteins.

The TCR has no intrinsic protein tyrosine kinase (PTK) activity; however, members of both the Src family and the Syk/ZAP-70 family of PTKs are implicated in the functioning of antigen receptors[4]. Current evidence indicates that Src family kinases phosphorylate the ITAMs of the TCR[4]. Zeta-associated protein (ZAP)-70 then associates with the doubly phosphorylated ITAMs of the ζ- and CD3 ε-chains through its Src homology-2 (SH2) domains[5], and is itself phosphorylated during early T-cell activation[6]. ZAP is a protein tyrosine kinase of relative molecular mass (M_r) 70K that exists exclusively in T cells and natural killer cells[7] and is important for T-cell activation. Genetic alterations in the ZAP-70 gene that cause loss of expression of ZAP-70 in humans prevent antigen activation of $CD4^+$ T cells, inhibit maturation of $CD8^+$ T cells, and lead to severe combined immunodeficiencies[8,9]. The binding of ZAP-70 to the TCR is believed to be essential for signal transduction, as peptides that block the association of ZAP-70 with the ζ-chain also inhibit T-cell signalling events[10].

ZAP-70 consists of two SH2 domains that are connected by a 65-residue segment (the inter-SH2 region) and are followed by a second connecting region and a catalytic domain[7]. Interaction of ZAP-70 with the TCR requires that both of its SH2 domains are present and functional, and that both tyrosines within the ITAM are phosphorylated[11-13]. SH2 domains play an integral role in intracellular signal transduction by mediating protein–protein interactions through specific recognition of tyrosine-phosphorylated proteins[14,15]. Selectivity by SH2 domains is dependent upon recognition of residues immediately carboxy-terminal to the phosphorylated tyrosine (pY + *n*). The three-dimensional structures of several isolated SH2 domains (both liganded and unliganded)[16-23], as well as one SH3–SH2 complex[24] and one SH3–SH2–SH3 protein[25], have been determined by X-ray crystallography or nuclear magnetic resonance.

Here we report the X-ray crystal structure of the tandem SH2 domains of human ZAP-70 (ZAP-NC) in complex with a doubly tyrosine-phosphorylated 19-meric peptide that is derived from the sequence of the first ITAM of the ζ-subunit (ζ_1) of the TCR at 1.9 Å resolution. The structure determination is summarized in Table 1 legend. The ZAP-NC:ζ_1 complex involves an extensive array of contacts between the peptide and both SH2 domains. The 65-residue inter-SH2 region exists as a coiled coil of α-helices and assists in the formation of an interface between the two SH2 domains. Within this interface, both SH2 domains contribute to the recognition of phosphotyrosine in the second pYXXL motif. This study provides structural insights into the Syk/ZAP family of PTKs and describes an intracellular component of the TCR.

General topology

The first 259 residues of ZAP-70 consist of two SH2 domains that are connected by a helical region. The overall fold is Y-shaped (Fig. 1*b*): the SH2 domains make up each upper branch, and the intervening 65 residues form the stem of the Y. The fragment of ZAP-70 used in the crystal structure determination terminates before the kinase domain; we therefore refer to the two SH2 regions as ZAP-N and ZAP-C, for ZAP N-terminal SH2 and ZAP C-terminal SH2, respectively. There is high structural similarity between each of the SH2 domains and those previously reported, such as $p60^{v\text{-}src}$ (ref. 16) and $p56^{lck}$ (ref. 19). Each of the individual ZAP SH2 domains possesses a central antiparallel β-sheet that is flanked by two α-helices. The inter-SH2 region begins as a β-strand that is a continuation of the central sheet of ZAP-N. This is followed by two antiparallel α-helices that form a coiled coil. The two SH2 domains are side by side, with an angle of ~52° between the central β-sheets.

ζ_1 is extended over both faces of the SH2 domains, straddles both central β-sheets, and makes extensive contacts with the protein surface. The binding orientation is head to tail, that is, the amino terminus of the peptide is in contact with the C-terminal SH2 domain. Binding of phosphorylated peptides to

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individual SH2 domains has been described as reminiscent of a 'socket and plug'[20], where the phosphotyrosine (pY) and the pY+3 residues are the prongs. This general arrangement is also present for each pYXXL contact with ZAP-C and ZAP-N. The peptide segment that separates the two pYXXL motifs is largely in contact with ZAP-C. The C-terminal phosphotyrosine is bound in a pocket that is formed by contributions from both SH2 domains (Fig. 1*a*, *c*). The nomenclature defined previously[19] for structural features of SH2 domains is used here for clarity (Fig. 2). Both BC loops of ZAP-NC are extended.

The inter-SH2 domain

The inter-SH2 spacer begins in a type II reverse turn followed by a long β-strand that forms an extension to the central β-sheet of ZAP-N. This segment is followed by a five-turn α-helix (designated helix C) that extends away from both SH2 domains, forming the stem of the overall Y shape of ZAP-NC. This helix is followed by a turn and a second α-helix (helix D) that curves around the axis formed by helix C. Helix D is distorted by two breaks and a short 3_{10}-helix. Helices C and D both make several hydrophobic contacts to ZAP-C. These antiparallel helices form a coiled coil, with direct contact between several hydrophobic residues forming its core. Several water-mediated hydrogen bonds exist between helix D and ZAP-C.

Binding of the complexed ζ_1 peptide

The bound conformation of ζ_1 is extended, although nearly one full α-helical turn exists between residues ζ Asn 8 and ζ Arg 12 (Fig. 1*c*). All residues of ζ_1, except for ζ Gly 10, are in contact with ZAP-NC. The area of the peptide–protein interface is over 1,300 Å^2. Although this interface area is typical for protein–protein interactions, the nature of the contacts is different from those generally observed[26]. For example, interfaces in antibody–antigen complexes and protease:protein-inhibitor complexes usually contain few bridging water molecules. The interaction of ZAP-NC with ζ_1 includes 21 bridging water molecules. Most of the contacts in protein–protein interfaces are usually classified as hydrophobic. In contrast, half of the contacts between ZAP-NC and ζ_1 are due to direct hydrogen bonds. The total number of contacts observed is considerably larger than is observed for protein–protein structures of similar interfacial areas.

Binding of motif-1 (-pYNEL-)

The N-terminal pYXXL motif of ζ_1 is associated exclusively with ZAP-C. The first two residues of ζ_1 are largely involved in intrapeptide interactions. The single contact between ζ Leu 3 and ZAP-NC, a hydrogen bond between its main-chain carbonyl and Arg 170, is typical of the pY−1 residue of peptides bound to SH2 domains.

The pocket for ζ pTyr 4 is formed by residues from helix A, strands B, C and D, and the BC loop. Hydrophobic contacts involve residues from βD, from which His 210, Tyr 211 and Leu 212 form one edge of the pTyr cavity. The side chain of Leu 212 is twisted away from the pTyr ring and is packed against Trp 131 from a symmetry-related molecule. This neighbouring Trp, which constitutes the only intermolecular crystal contact with any ζ_1 residue, is also in hydrophobic contact with ζ pTyr 4. Direct hydrogen-bonding contacts to the phosphate are made by only three residues (Fig. 3*a*). Arg 170 (αA) and Arg 190 (βB) interact through their terminal nitrogens. Arg 192 is the only residue in the region of the BC loop that is of sufficient length for direct hydrogen bonding to the phosphate group and interacts via its Nε. The BC loop is extended, so the pTyr binding region resembles a deep groove that continues toward the AA loop. Five water molecules with very strong density and low temperature factors exist in this region and are part of a large hydrogen-bonding network.

As is typical for complexes with SH2 domains[19–21], the pY+1 and pY+2 residues are extended along the surface of the protein. The pocket that surrounds ζ Leu 7 (pY+3) is very deep and is formed by residues from βD, the EF loop, helix B and the BG

TABLE 1 Statistics for data collection, phase determination and refinement

Data collection	Resolution (Å)	Reflections (N)	Completeness (%)	R_{sym} (%)*	R_c†	Figure of merit	Phasing power (20–2.8 Å)‡
TML	25–2.5	15,506	98.3	6.3			
SeMet TML	25–1.9	23,978	98.9	4.9	0.71	0.50	1.38

Refinement

Model 272 residues, 113 water molecules, 1 lead, 3 selenium atoms

Resolution	Reflections ($F>2\sigma$)	R-value (%)§	Free R-value (%)‖	R.m.s. deviations Bonds (Å)	Angles (°)
10–1.9	23,697	20.9	25.5	0.006	1.58

Details of peptide synthesis and the expression and purification of ZAP-NC will be published elsewhere. The peptide (NQLpYNELNLGRREEpYDVLD) was synthesized by automated solid-phase synthesis. ZAP-NC (residues 1–259) was expressed as a glutathione S-transferase (GST) fusion protein and cleaved with thrombin. **Crystallization.** ZAP-NC complexed with doubly phosphorylated ζ_1 and treated with trimethyllead acetate (TML) was crystallized by vapour diffusion in hanging drops containing 13.5 mg ml^{-1} complex and 10% PEG 4000, 50 mM sodium citrate, 100 mM ammonium acetate, 0.005% sodium azide and 20 mM dithiothreitol, pH 6.2, over reservoirs of 20% PEG 4000 and 20 mM dithiothreitol. The crystals are monoclinic ($P2_1$, $a=50.11$, $b=63.37$, $c=54.00$ Å, $\beta=114.44°$) with one molecule per asymmetric unit. **Data collection.** Diffraction data were collected at room temperature with a Rigaku R-AXIS II area detector with graphite monochromated Cu K_α X-rays. Diffraction data were collected as 2° oscillation images and reduced to integrated intensities using DENZO[42]. Scaling parameters for each image were calculated with ROTAVATA[43] and applied with AGROVATA[43]. **MIR analysis and refinement.** Selenomethionyl (SeMet) ZAP-NC was crystallized with TML under the same conditions and data collected. Positions of the lead and selenium atoms were determined from the difference Patterson function. Anomalous dispersion measurements were included for both datasets. Heavy-atom parameters were refined, and phases were obtained at 2.8 Å using MLPHARE[43]. The MIR phases were improved with the program DM[43] with a combination of solvent flattening/histogram mapping and phase extension to 2.0 Å. Electron-density maps with MLPHARE and DM phases were calculated, and the polypeptide chain model was built with the program O[44]. SIGMAA[43] was used to perform several cycles of phase combination using partial model and experimental phases. Least-squares refinement with simulated annealing was done using X-PLOR[45]. The current model has all residues from Asp 3 to Asn 256 of the protein, all 19 peptide residues, and 113 water molecules, plus one lead and three selenium atoms. TML is bound to Cys 117.

* $R_{sym}=\sum|I_i-\langle I\rangle|/\sum I_i\times 100$.

† $R_c=\sum\|F_{ph}\pm F_p|-F_{hcalc}|/\sum F_{ph}\pm F_p|$ for centric reflections.

‡ Phasing power, F_h/E, where E=r.m.s. lack of closure error.

§ $R_{value}=\sum\|F_{obs}|-|F_{calc}\|/\sum|F_{obs}|\times 100$.

‖ Subset of data (10%) was excluded from refinement and used for free R-value calculation. All data with $F>2\sigma$ were used for refinement.

loop. Owing to the size of this pocket, ζ Leu 7 is contacted directly by only 5 residues: Tyr 211, Ile 223, Gly 226, Gly 245 and Leu 246. The depth of this pocket is partly due to the presence of a leucine in helix B that is occupied by a tyrosine in many other SH2 domains[21]. A second contribution to the overall shape of the pY+3 pocket is provided by a repositioning of the β-turn in the EF loop. In complexes of isolated SH2 domains, this loop is involved in forming the steep solvent-exposed wall of the pocket. In ZAP-C, the EF loop slides toward strand D to allow the remainder of the peptide to continue on its path toward ZAP-N.

Binding of intermotif (-NLGRREE-)

The peptide segment that separates the pYXXL motifs in ζ_1 consists of seven amino acids that make the bulk of their contacts to ZAP-C. Because nearly a full turn of an α-helix begins at ζ Asn 8 and continues to ζ Arg 12, many contacts for this sequence are intrapeptide. ζ Asn 8 and ζ Arg 12 both contact ZAP-C by several direct and water-mediated hydrogen bonds. The side chains of ζ Leu 9 and ζ Arg 12 close off the pY+3 pocket of ZAP-C.

Two glutamate residues complete this segment of ζ_1. As the pY−1 and pY−2 residues of the second pYXXL motif, they constitute the first contacts to ZAP-N. ζ Glu 13 is involved in a direct hydrogen bond through its side-chain carboxyl to the side-chain amino group of Lys 242 (ZAP-C αB), which is an integral part of the phosphotyrosine pocket of the N-terminal SH2 domain. ζ Glu 13 also maintains van der Waals contact to Lys 242, as well as to Tyr 238 (ZAP-C αB) and Arg 17 (ZAP-N αA), which also contribute to the N-terminal pY pocket. ζ Glu 14 maintains the characteristic pY−1 contacts.

Binding of motif-2 (-pYDVL-)

The most remarkable and unique feature of the complex between ZAP-NC and ζ_1 is the recognition pocket of ζ pTyr 15, which is composed of residues from both SH2 domains (Fig. 3*b*, *c*). The impinging of ZAP-C on ZAP-N sequesters ζ pTyr 15 in a deep tunnel. The side chain of ζ pTyr 15 makes van der Waals contacts to Arg 41 (BC loop), Val 47 (βC), His 58 (βD) and Pro 60 (βD). The side chain of Arg 17 is positioned over the aromatic ring of ζ pTyr 15, forming an amino-aromatic contact in addition to bridging the carbonyl of the pY−1 residue to the phosphate oxygens of ζ pTyr 15. The phosphate group is closely associated with the side chains of Tyr 238 and Lys 242 (both donated by ZAP-C αB), Arg 17 (αA) and Arg 37 (βB), forming a total of six direct hydrogen bonds. Six water-mediated hydrogen bonds exist between the phosphate group and Arg 17 (αA), Cys 39 (βC), Leu 40 (BC loop), Arg 41 (BC loop) and Lys 242 (ZAP-C αB). Mutagenesis experiments are required to determine the relative importance of each residue in this interface. Four water molecules contribute to this extensive network; their presence may be a consequence of the intrusion of ZAP-C onto residues of the BC loop.

The pY+1 and pY+2 residues (ζ Asp 16 and ζ Val 17) make contacts that are characteristic of these positions in other SH2 complexes[19-21]. ζ Leu 18 resides in a hydrophobic pocket that is of similar dimension to the pY+3 pockets observed in high-affinity peptide complexes with Src family SH2 domains. Contact with several hydrophobic residues is evident: βD contributes Phe 59; interaction with the EF loop involves Ile 71, Ala 72, Gly 73 and Gly 74; helix B presents Tyr 87; and the BG loop makes contact by means of Gly 93 and Leu 94.

Unlike the extensive contact area between ζ_1 and ZAP-NC, the total interaction area between ZAP-N and ZAP-C is small, measuring only ~200 Å^2. The interface that is exclusively between ZAP-N and ZAP-C consists mostly of water-mediated hydrogen bonds, although van der Waals contacts do exist (Fig. 4). Given that the total interface that is exclusive to the two SH2 domains may not exist without the peptide, the inter-SH2 spacer is likely to stabilize the appropriate orientation for tandem binding by allowing only minor displacements. In isoelectric focusing gels, uncomplexed ZAP-NC exists as multiple bands which collapse into a single band when ζ_1 is added (J.L.K. and M.H.H., unpublished observation). This microheterogeneity observed with uncomplexed ZAP-NC may be indicative of conformational variability.

Comparison with other complexes

Despite the low sequence identity (33%) between ZAP-N and ZAP-C, the similarity in overall fold is notable. Side-chain positions are remarkably well conserved between the two domains. The overall backbone root mean square (r.m.s.) deviation is 1.07 Å; the same measurement for ZAP-N or ZAP-C to Src family SH2 domains is typically 1.50 Å, although the percentage of sequence identity is similar. As reported for previous structures of SH2 domains[19,20,23], the loop regions display the largest positional variance.

Although each pYXXL motif of ζ_1 resides in a similar backbone conformation in the complex, the orientation of the phosphotyrosines varies between ZAP-N and ZAP-C. The aromatic ring of ζ pTyr 4 superimposes remarkably well with the pTyr in both Lck[19] and v-Src[20]. For ζ pTyr 15, however, the ring is repositioned 0.7 Å towards the guanidinium of Arg 17 and slips 0.8 Å away from strand D. This is probably due to the direction taken by ζ_1 as it moves into the ZAP-C domain, as well as the strong hydrogen-bonding interactions between the phosphate group and Tyr 238 and Lys 242 on ZAP-C. Both pTyr pockets of ZAP-NC are large enough for several water molecules to be included; this enlargement relative to other SH2 domains is due to the repositioning of the BC loop. The extended position for the BC (phosphotyrosine binding) loop observed for both SH2 domains of ZAP-70 has been observed previously for uncomplexed SH2 domains, and has been described as a hinge in the binding of tyrosine phosphorylated and phosphonated peptides[20,23]. Although the loop is reorientated, the internal conformation for the BC loop is strongly maintained.

Finally, in comparison with all other crystal structures of complexes of SH2 domains, a significant number of water molecules are present in ZAP-NC. Several are involved in bridging phosphotyrosine to the protein. Additionally, a large number of buried water molecules exist in all interfaces.

Biological significance

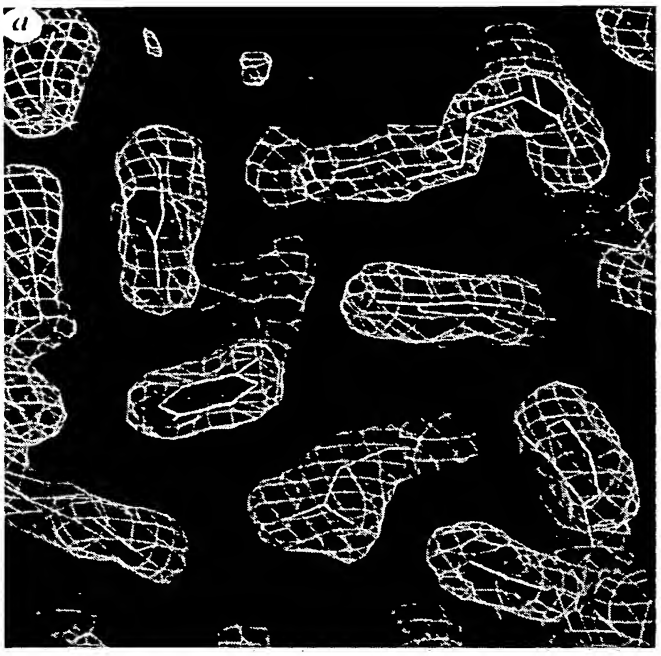
The structure of the tandem SH2 domains of ZAP-70 in complex with a component of the ζ-chain of the TCR provides a view at the molecular level into the intracellular machinery of the TCR (Fig. 5). This structure suggests that the SH2 domains do not function independently, and that interactions between the domains are critical in the recognition of the TCR. The structure is key to the interpretation of genetic and biochemical data, and provides a framework for exploring the mechanism of action of ZAP-70.

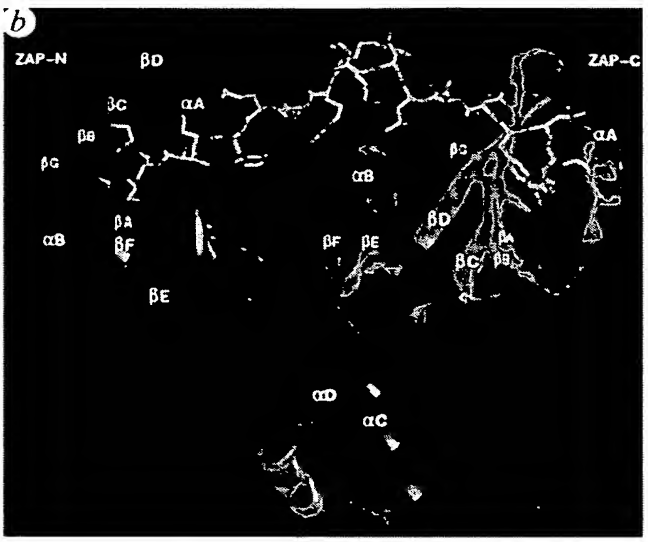
The tandem SH2 domains of ZAP-70 exhibit high selectivity for the phosphorylated ζ- and ε-subunits of the TCR, but isolated SH2 domains from other proteins bind to many tyrosine-phosphorylated proteins in total cell extracts[5]. Although ZAP-NC exhibits high affinity for doubly phosphorylated ITAMS and selectivity for the ζ- and ε-chains, the individual SH2 domains of ZAP-70 have not been found to bind appreciably to phosphorylated peptides[5]. In addition, ZAP-NC binds to monophosphorylated ITAM-based peptides with affinities that are 100–1,000 times lower than for the corresponding doubly phosphorylated ones[11,13,27]. The selectivity of ZAP-70 for doubly phosphorylated ITAM sequences appears to be a consequence of multiple structural features. The distance between the two pYXXL motifs of the ζ- or ε-chain provides properly spaced partners for a pair of SH2 domains that are tethered in close

association by an inter-SH2 coiled coil. Consequently, high-affinity binding is due to the dramatic entropic advantage that stems from the bidentate interaction. The structural manifestation of this is also the most remarkable feature of the complex between ZAP-NC and ζ_1, that is, the convergence of residues from both SH2 domains to enmesh pTyr 15.

It has so far been assumed that SH2 domains adopt their native fold when extracted from their natural molecular context and possess their full ability to recognize and bind to phosphorylated proteins[15]. Here we present structural evidence that the N-terminal SH2 domain of ZAP-70, if expressed in isolation, is incomplete. The groove-like nature of the pY pocket of ZAP-C suggests that this domain may also require contributions from neighbouring domains or proteins.

Studies involving mutational alterations in ITAM sequences of TCR subunits show that one additional residue between





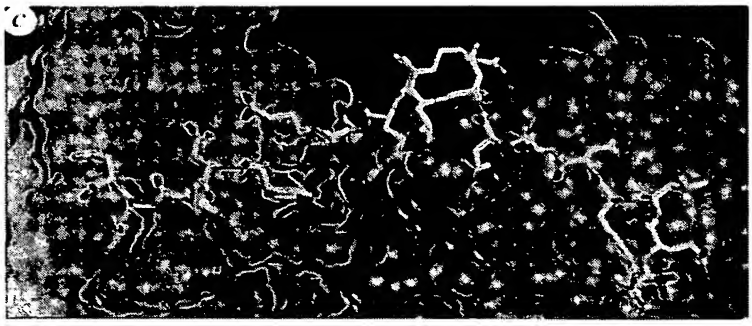


FIG. 1 Electron density and overall topology of ZAP-NC + ζ_1. *a*, Electron-density map near the interface between the two SH2 domains. The map was calculated with $2|F_o| - |F_c|$ coefficients for data between 15.0 and 1.9 Å, contoured at 1.0σ. *b*, Backbone ribbon representation of the overall fold of the complex. Secondary structural elements are labelled according to the convention for SH2 domains[19]. The N-terminal SH2 domain is coloured red, the inter-SH2 domain region orange, and the C-terminal SH2 domain blue. The ζ_1 peptide is shown explicitly; its carbon atoms are yellow, the oxygens red, nitrogens blue, and the phosphorus atoms of phosphotyrosine residues orange. *c*, Bound conformation of the ζ_1 peptide. The N terminus of the peptide is at the right-hand side of the figure. The peptide consists of 19 residues, starting at residue 48 of the mature ζ-subunit of the TCR. Phosphotyrosines are at relative positions 4 and 15. The peptide is illustrated along with the solvent-accessible surface for the heavy atoms of ZAP-NC, computed using MS[46]; each SH2 domain is represented by a different colour. ZAP-N is illustrated in cyan and ZAP-C in green. The pYXXL motifs are separated by an S-shaped segment of peptide. Pockets for each pY and pY + 3 residue are visible. Note that the N-terminal ζ_1 residues contribute to the enclosure of ζ pTyr 4. Parts *b* and *c* were generated with Sybyl (Tripos).

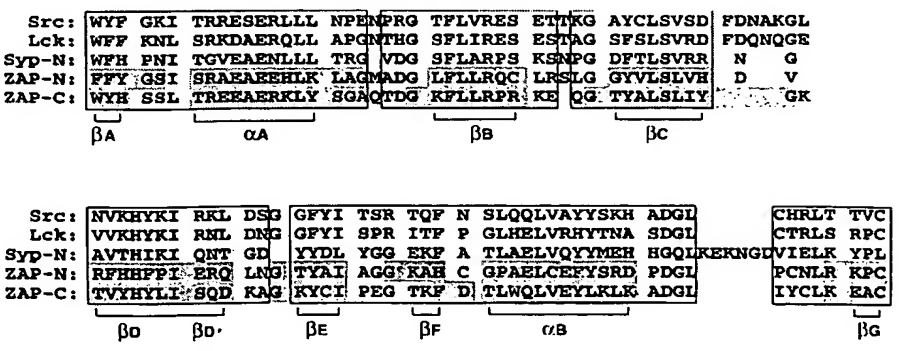


FIG. 2 Sequence alignments for selected SH2 domains[36]. Boxed areas indicate segments used for measuring the 'full' backbone r.m.s. deviation reported in the text; unboxed areas are excluded from the calculation owing to the presence of gaps for one or more of the sequences. Notation below the alignments indicate structurally conserved regions, and use the nomenclature reported previously[19]. Shaded regions indicate the secondary structural elements in ZAP-NC. Src, avian Src; Lck, human p56^{lck}; Syp-N, N-terminal SH2 of murine Syp phosphatase (PTP1D); ZAP-N and ZAP-C, N- or C-terminal SH2 of human ZAP-70.

pYXXL motifs is tolerated, but a deletion of two amino acids drastically reduces ZAP-70 binding and eliminates production of interleukin (IL)-2[11]. Therefore the distance between the two pYXXL motifs is important for association and signalling. The relative contribution of each residue of the ITAM was explored by alanine scanning[28]. Only the replacement of each pY or pY+3 residue eliminates signalling completely, as measured by IL-2 production. Apart from these residues, the specific sequence of the ITAM is less important for binding than the distance between pYXXL motifs.

The inter-SH2 region constrains the SH2 domains of ZAP-70 within a distance that allows their association. However, because a significant portion of the antiparallel helices is directed away from the SH2 domains, we speculate that this region may also be important for inter- or intramolecular interactions that regulate the kinase activity of ZAP, dimerization during TCR clustering, or association with other cellular proteins. Coiled coils are commonly involved in protein–protein interactions[29]. The inter-SH2 domain may inhibit the catalytic activity of ZAP-70 through intramolecular interactions that are relieved upon binding of the SH2 domains to the ITAM. Experiments with phosphatidylinositol-3-OH kinase (PI(3)K)[30] and Syk[31] support this model. Activation of PI(3)K is induced *in vitro* by a phosphotyrosine peptide from the platelet-derived growth factor (PDGF)-β receptor[30]. Similarly, phosphorylated ITAM peptides derived from the γ-subunit of the IgE receptor increase Syk kinase activity by five- to tenfold[32,33]. There is also precedent for involvement of such inter-SH2 regions in intermolecular associations. The inter-SH2 region of the p85 subunit of PI(3)K, which is predicted to form a coiled coil, is necessary and sufficient for

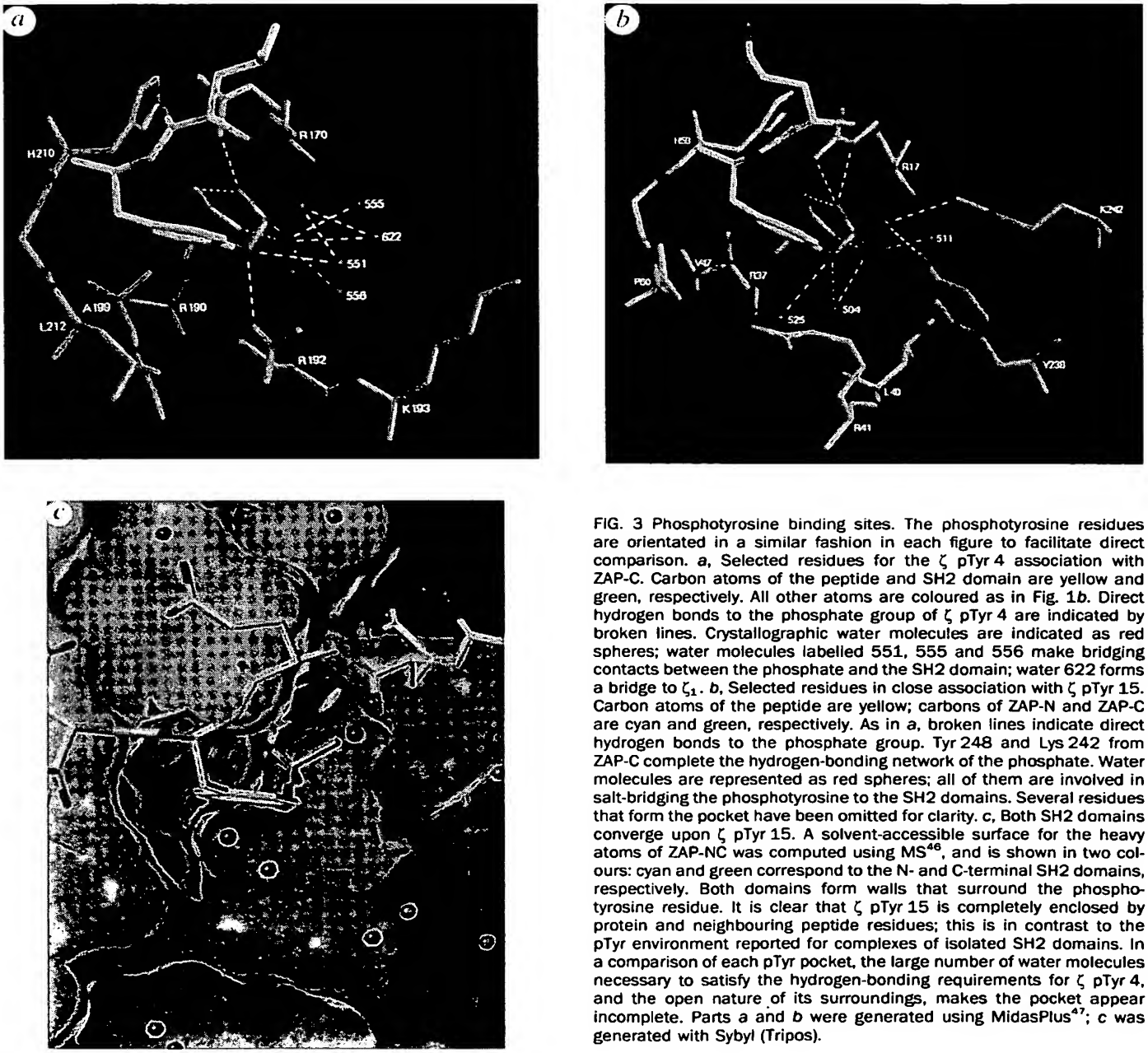


FIG. 3 Phosphotyrosine binding sites. The phosphotyrosine residues are orientated in a similar fashion in each figure to facilitate direct comparison. *a*, Selected residues for the ζ pTyr 4 association with ZAP-C. Carbon atoms of the peptide and SH2 domain are yellow and green, respectively. All other atoms are coloured as in Fig. 1*b*. Direct hydrogen bonds to the phosphate group of ζ pTyr 4 are indicated by broken lines. Crystallographic water molecules are indicated as red spheres; water molecules labelled 551, 555 and 556 make bridging contacts between the phosphate and the SH2 domain; water 622 forms a bridge to ζ_1. *b*, Selected residues in close association with ζ pTyr 15. Carbon atoms of the peptide are yellow; carbons of ZAP-N and ZAP-C are cyan and green, respectively. As in *a*, broken lines indicate direct hydrogen bonds to the phosphate group. Tyr 248 and Lys 242 from ZAP-C complete the hydrogen-bonding network of the phosphate. Water molecules are represented as red spheres; all of them are involved in salt-bridging the phosphotyrosine to the SH2 domains. Several residues that form the pocket have been omitted for clarity. *c*, Both SH2 domains converge upon ζ pTyr 15. A solvent-accessible surface for the heavy atoms of ZAP-NC was computed using MS[46], and is shown in two colours: cyan and green correspond to the N- and C-terminal SH2 domains, respectively. Both domains form walls that surround the phosphotyrosine residue. It is clear that ζ pTyr 15 is completely enclosed by protein and neighbouring peptide residues; this is in contrast to the pTyr environment reported for complexes of isolated SH2 domains. In a comparison of each pTyr pocket, the large number of water molecules necessary to satisfy the hydrogen-bonding requirements for ζ pTyr 4, and the open nature of its surroundings, makes the pocket appear incomplete. Parts *a* and *b* were generated using MidasPlus[47]; *c* was generated with Sybyl (Tripos).

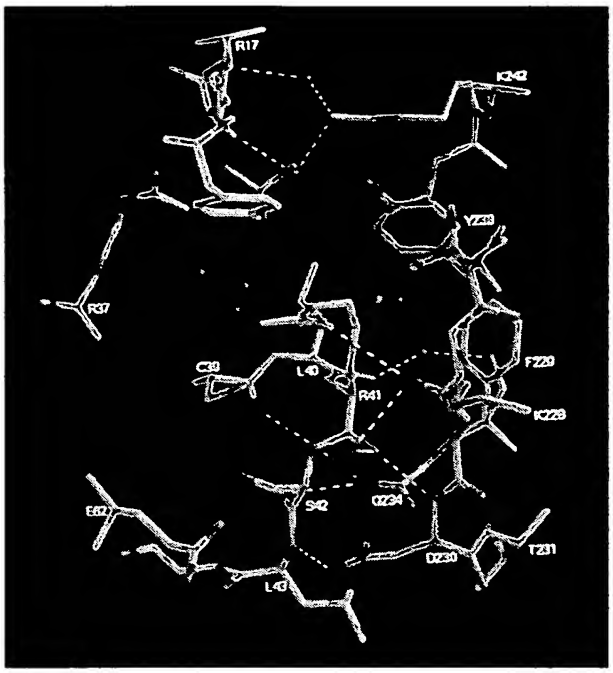


FIG. 4 The interface between the SH2 domains. Following the convention established in Fig. 3, carbon atoms for ZAP-N, ZAP-C and the peptide are illustrated in cyan, green and yellow, respectively. Each SH2 domain contributes nine residues to the interface. An extensive network of hydrogen bonds is present; the most prominent contacts are indicated by broken lines. Interactions involving ζ pTyr 15 are described in Fig. 3*b*. Several hydrophobic contacts also exist, of which the most provocative is the protrusion of Arg 41 (ZAP-N BC loop) into a small hydrophobic depression formed by Phe 229 (ZAP-C βF), Tyr 238 (ZAP-C αB) and the side chain of Thr 227 (ZAP-C EF loop). This is most evident in the density map of Fig. 1*a*. Arg 41 also has three hydrogen-bonding contacts to ZAP-C. This is the first of three residues in the BC loop of ZAP-N that form an artificial parallel sheet with strand F in ZAP-C. Only one of the three hydrogen-bonding contacts involves main-chain atoms exclusively. This figure was generated using MidasPlus[47].

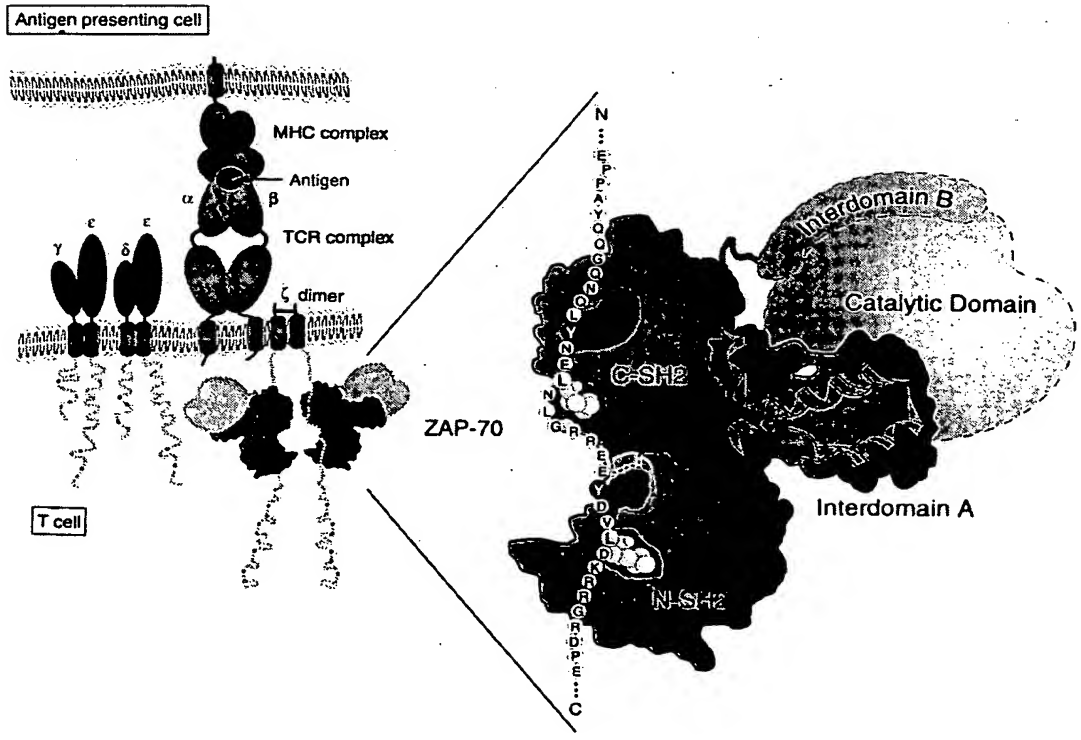


FIG. 5 Schematic view of ZAP-70 bound to ζ_1 of the activated TCR. Left, activation of T cells is initiated by association of the TCR with a peptide antigen bound to the major histocompatibility complex (MHC) on an antigen-presenting cell. TCR–MHC association stimulates phosphorylation of TCR subunits on tyrosines (shown in red) within the ITAMs. ZAP-70 binds to the phosphorylated ITAM by means of its SH2 domains (residues 1–259). The positions of the other domains of ZAP-70, referred to as interdomain B (residues 260–310) and catalytic domain (residues 311–620), have not been determined, and their positions as shown are hypothetical. Two ZAP-70 molecules could bind to the activated TCR complex, as the ζ-subunit is present as a disulphide-linked dimer. Right, schematic representation of the complex between the SH2 domains of ZAP-70 in complex with the doubly phosphorylated ζ_1 ITAM. The SH2 domains of ZAP-70 make extensive contacts with ζ_1. The primary determinants of binding are the phosphotyrosine (red) and leucine (yellow) residues of two pYXXL sequences within an ITAM. The structure reveals a unique binding pocket for the pY of the C-proximal pYXXL motif in the interface between the two SH2 domains. In addition, the crystal structure reveals that interdomain A forms a coiled coil. This domain may participate in positioning the two SH2 domains for association with ITAMs, and in communicating structural changes to interdomain B and/or the kinase domain upon receptor engagement.

interaction of p85 with the p110 catalytic subunit of PI(3)K[34]. The inter-SH2 region could be involved in interactions with proteins that regulate ZAP-70, such as Lck or Fyn, or that are substrates for ZAP-70. As there is at least one tyrosine in the inter-SH2 region that is phosphorylated by Lck *in vitro*[35], such interactions could involve SH2 domains from other proteins.

We anticipate that Syk will also exhibit these structural features as a result of the functional similarities and its sequence identity of 57%. Syk is expressed in several types of haematopoietic cells, and functions in mast cells and B cells by binding to ITAM sequences in the cytoplasmic domains of IgE and B-cell receptors, respectively[31]. Most of the residues in ZAP-NC that contact pTyr 15 are conserved in the corresponding positions in Syk[36]. The N-terminal SH2 domain of Syk does not bind to phosphotyrosine ligands or to phosphotyrosine affinity columns[37], which suggests that this phosphotyrosine site also requires the C-terminal SH2 domain for it to form a complete pocket. It is known that doubly phosphorylated peptides derived from the γ ITAM of the IgE receptor induce Syk activation[32] We therefore infer that this complex of ZAP-NC with ζ_1 represents the conformation of the SH2 domains in the activated kinase.

ZAP-70 is an attractive target for the development of potent immunosuppressive drugs. It is required for T-cell-mediated immune responses in humans, and its loss does not affect other tissues[8]. Thus ZAP-70 antagonists would inhibit T-cell function specifically and avoid the well-documented toxicity of currently used immunosuppressives. Tacrolimus (FK506) and cyclosporin[38 41] exhibit side effects that limit their application largely to organ transplant rejection[38]. Therefore new immunosuppressive drugs with lower toxicity are needed to expand the routine use of such therapies to autoimmune diseases.

One approach to the inhibition of T-cell activation is to develop non-peptidic molecules that prevent ZAP-70 association with the TCR. Because both ZAP-70 SH2 domains are required for TCR coupling, a membrane-permeable inhibitor that binds with high affinity to either SH2 domain of ZAP-70 or that alters the structure of the interface should inhibit ZAP-70 function in activated T cells. The crystal structure provides the molecular details of ZAP-70 interactions with the TCR. The unique structural features of each SH2 domain and the unanticipated interfaces can be exploited for structure-based design of specific ZAP-70 ligands and structurally biased combinatorial libraries. □

Received 1 June; accepted 13 July 1995.

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ACKNOWLEDGEMENTS. We thank S. L. Schreiber, J. S. Brugge and M. Weigele for comments and discussions. Coordinates will be deposited in the Brookhaven Protein Data Bank.

EXHIBIT H

AMENDMENT IN RESPONSE TO JULY 22, 2009 OFFICE ACTION AND PETITION FOR ONE-MONTH EXTENSION OF TIME

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

Docket No. 0575/67489-PCT-US/JPW/BJA/MJP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Stephen P. Goff and Guangxia Gao

International Application No. : PCT/US2004/026162

Serial No. : 10/568,396 Examiner : Liu, S.

Filed : August 31, 2006 Art Unit : 1656

For : ZAP PROTEIN AND RELATED COMPOSITIONS AND METHODS

30 Rockefeller Plaza
20th Floor
New York, New York 10112

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. §1.132

I, Stephen P. Goff, hereby declare that:

1. I am the Stephen P. Goff named as an inventor on the above-identified application.

2. I understand that the claims attached hereto as Exhibit 1 are pending in the above-identified application and have been rejected on the basis of Gao et al. *Science* 2002 September 6; 297(5587):1703-6 (hereinafter Gao et al.).

3. I am a named author on Gao et al. Guangxia Gao and Xuemin Guo are co-authors.

4. Guangxia Gao is named as a coinventor on the above-identified

application. He and I conceived of the invention recited in both the pending claims (Exhibit 1) and the claims attached hereto as Exhibit 2.

5. Xuemin Guo performed experiments described in Gao et al. under Guangxia Gao's or my direction and supervision. He did not contribute to the conception of the invention recited in either the pending claims (Exhibit 1) or the claims attached hereto as Exhibit 2.

I declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Dated: 12/3/09

Stephen P. Goff

EXHIBIT 1

DECLARATION UNDER 37 C.F.R. §1.132

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

Exhibit 1: Pending claims as of April 8, 2009 Amendment

1. (Original) An isolated ZAP protein.

2. (Original) The protein of claim 1, wherein the protein is a human ZAP protein.

3. (Original) The protein of claim 1, wherein the protein is a rat ZAP protein.

4. (Original) The protein of claim 1, wherein the protein is a mouse ZAP protein.

5. (Original) The protein of claim 1, wherein the protein comprises the amino sequence set forth in SEQ ID NO: 1.

6. (Original) The protein of claim 1, wherein the protein has deleted from it a region which causes protein instability.

7. (Original) The protein of claim 6, wherein the region is the WWE region.

8-36. (Canceled)

37. (Currently Amended) A method for increasing the amount of ZAP protein in a subject's cells which comprises administering to the subject an amount of ~~ZAP protein~~ the protein of claim 1 effective to increase the amount of ZAP protein in the subject's cells.

38-56. (Canceled)

57. (New) The method of claim 37, wherein the subject is a human.

58. (New) A method for increasing resistance to a virus in a subject which comprises administering to the subject an amount of the protein of claim 1 effective to increase the amount of ZAP protein in the subject's cells, so as to thereby increase resistance to the virus in the subject.

59. (New) The method of claim 58, wherein the subject is a human.

60. (New) The method of claim 58, wherein the virus is an alpha virus.

61. (New) The method of claim 58, wherein the virus is West Nile virus.

EXHIBIT 2

DECLARATION UNDER 37 C.F.R. §1.132

Serial No. 10/568,396

Filed: August 31, 2006

Applicants: Stephen P. Goff and Guangxia Gao

Exhibit 2: Pending claims as of December 22, 2009 Amendment

1. (Currently Amended) An isolated mammalian Zinc-finger antiviral protein (ZAP) ~~ZAP~~ protein which:
   (a) comprises four CCCH-type zinc finger motifs; and
   (b) when present in a mammalian cell infected with a retrovirus, binds to RNA of the retrovirus, so as to inhibit replication of the retrovirus in the cell.

2-5. (Canceled)

6. (Original) The protein of claim 1, wherein the protein has deleted from it a region which causes protein instability.

7. (Currently Amended) The protein of claim 6, wherein the region is the WWE domain ~~region~~.

8-36. (Canceled)

37. (Withdrawn; Currently Amended) A method for increasing the amount of ~~ZAP~~ Zinc-finger antiviral protein (ZAP) protein in a subject's cells which comprises administering to the subject an amount of the protein of claim 1 effective to increase the amount of Zinc-finger antiviral protein (ZAP) ~~ZAP~~ protein in the subject's cells.

38-56. (Canceled)

57. (Withdrawn) The method of claim 37, wherein the subject is a human.

58. (Withdrawn; Currently Amended) A method for increasing resistance to a retrovirus ~~virus~~ in a subject which comprises administering to the subject an amount of the protein of claim 1 effective to increase the amount of Zinc-finger antiviral protein (ZAP) ~~ZAP~~ protein in the subject's cells, so as to thereby increase resistance to the retrovirus ~~virus~~ in the subject.

59. (Withdrawn) The method of claim 58, wherein the subject is a human.

60-61. (Canceled)